GLUT2 is a high affinity glucosamine transporter

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Abstract When expressed in *Xenopus* oocytes, GLUT1, 2 and 4 transport glucosamine with V_{max} values that are three- to four-fold lower than for glucose. The K_ms for glucosamine and glucose of GLUT1 and GLUT4 were similar. In contrast, GLUT2 had a much higher apparent affinity for glucosamine than for glucose ($K_m = 0.8 \pm 0.1$ mM vs. ~17–20 mM). Glucosamine transport by GLUT2 was confirmed in mammalian cells and, using hepatocytes from control or GLUT2-null mice, $HgCl_2$-inhibitable glucosamine uptake by liver was shown to be exclusively through GLUT2. These data have implications for glucosamine effects on impaired glucose metabolism and for structure–function studies of transporter sugar binding sites.

Key words: Glucosamine; GLUT; Hepatocyte; Metabolism

1. Introduction

Glucosamine (GlcN) can be produced by the hexosamine biosynthesis pathway and converted to uridine diphosphate-*N*-acetyl glucosamines. This activated sugar is used for *O*-linked glycosylation of several proteins, including transcription factors, RNA polymerase and nuclear pore proteins, leading to alteration in their biological activity [1–3]. Increased activity of the hexosamine pathway represents an important mechanism by which hyperglycemia causes insulin resistance [4–6]. This has been demonstrated, in particular, by overexpressing the rate-limiting enzyme of the hexosamine pathway, glutamine fructose-6-phosphate amidotransferase (GFAT), which catalyzes the conversion of fructose-6-phosphate to *N*-acetylglucosamine-6-phosphate, in different tissues. Transgenic overexpression of GFAT in muscles and adipose tissue leads to decreased glucose uptake, mostly because of a defect in the translocation to the plasma membrane of the glucose transporter GLUT4 [5,7]. When GFAT is overexpressed in mouse pancreatic β cells it induces hyperinsulinemia, peripheral insulin resistance and a reduction in insulin mRNA expression [8].

GlcN taken up by cells is phosphorylated into GlcN-6-phosphate by hexokinase, which then bypasses GFAT to enter the hexosamine pathway. Exposure of isolated adipocytes [7] or rat skeletal muscles [9] to GlcN leads to insulin resistance. In β cells, GlcN induces several dysfunctions by a mechanism which appears to involve oxidative stress rather than *O*-linked glycosylation [10]. Incubation of kidney cells with GlcN produces a transcriptional upregulation of transforming growth factor β, suggesting a possible involvement of the hexosamine pathway in diabetic nephropathy [11]. GlcN also induced platelet-derived growth factor-activated DNA synthesis in rat aortic smooth muscle cells, suggesting that it may be involved in the development of atherosclerosis [12]. In vivo studies showed that GlcN infusion also produces insulin resistance in both muscle and fat tissues in mice and rats [13–15]. In humans, it has been observed that acutely administered GlcN induces a mild dysfunction in pancreatic β cell secretion and, at higher doses, an impairment in glucose utilization under hyperglycemic/hyperinsulinemic conditions [16].

GlcN is important in the metabolism of all glycoproteins, including those in the cartilage, where it is required for the formation of the glycosaminoglycans. In association with collagen fibers, these molecules are responsible for the resilience of the cartilage to deformation [17]. Destruction of joint cartilage occurs in osteoarthritis, and several studies have shown that GlcN ingestion is beneficial for this disease [18–21]. This compound may exert its positive effects by increasing the production of aggrecan by chondrocytes [22].

Exogenous supplementation of GlcN can thus mediate positive effects on osteoarthritis but adverse effects on glucose homeostasis. Several studies have used GlcN infusion to stimulate the hexosamine pathway. Here we show that three members of the GLUT family (GLUT1, 2, 4) can transport GlcN. However, whereas GLUT1 and GLUT4 have similar apparent affinities for glucose and GlcN, GLUT2 has a ~20-fold higher affinity for GlcN than for glucose.

2. Materials and methods

2.1. Microinjection and GlcN uptake

GLUT1 and GLUT4 (LL489/490AA mutant) cDNAs in the pXOV plasmid (provided by Dr. Mike Mueckler) and GLUT2 in the PSD5 plasmid were used for preparation of synthetic RNAs by in vitro transcription, as described [23]. Transport measurements were performed 3 days after RNA injection with groups of 8–12 oocytes. Uptake was initiated by incubating oocytes in 200 μl of MBS (NaCl 85 mM, KCl 1 mM, $NaHCO_3$ 2.4 mM, $MgSO_4$ 1 mM, $Ca(NO_3)_2$ 0.33 mM, $CaCl_2$ 0.55 mM, HEPES 4 mM) containing 10 μCi of D-[1-^{3}H]GlcN or D-[1-^{3}H]glucose (NEN Life Science) diluted in non-radioactive GlcN or glucose, respectively, at final concentrations ranging from 100 μM to 100 mM. Uptake was terminated after 15 min by washing the oocytes with ice-cold MBS containing $HgCl_2$. Individual oocytes were then dissolved individually in 5% sodium dodecylsulfate (SDS) and counted. Curves corresponding to the Michaelis–Menten equation were fitted to the data and transformed to Eadie–Hofstee plots for K_m and V_{max} determination.

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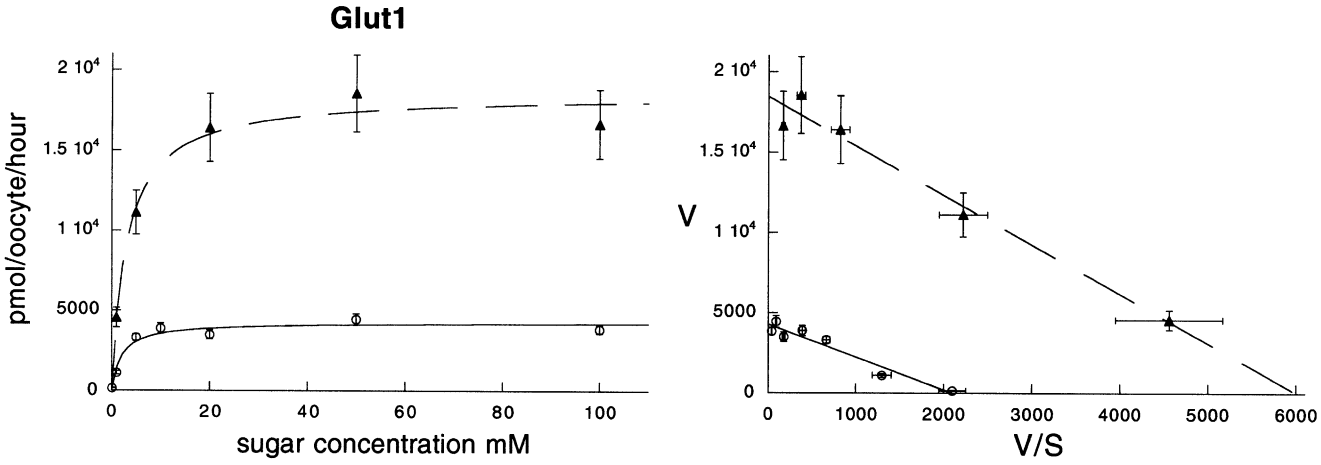
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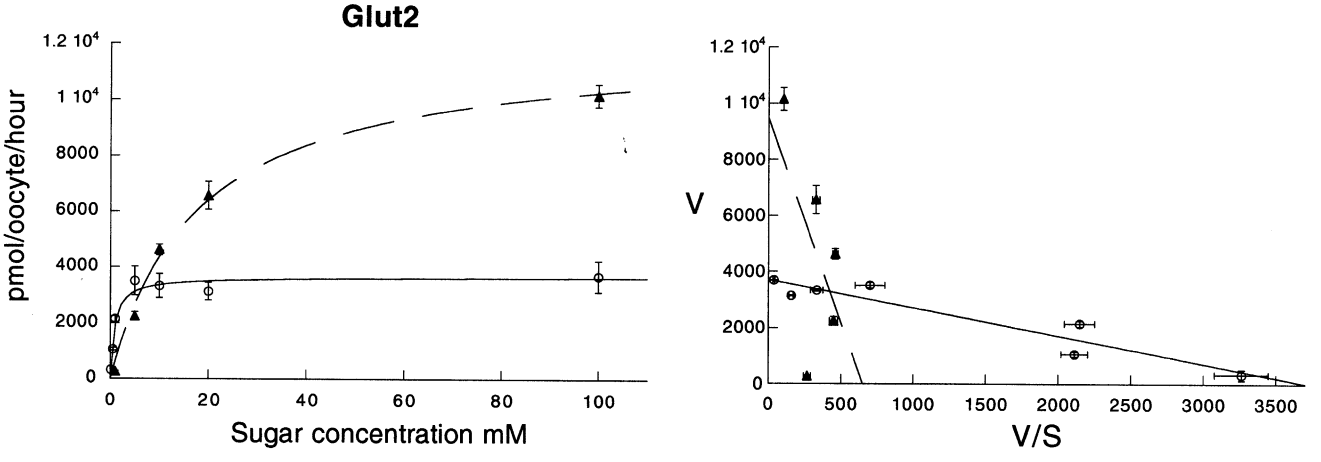
Glut2
pmol/oocyte/hour
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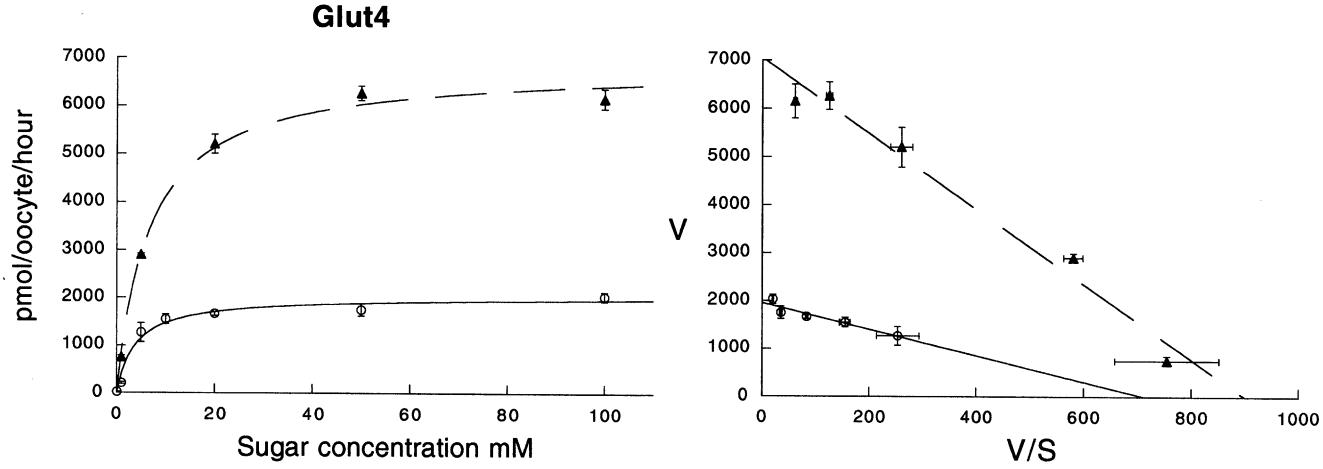
Glut4
pmol/oocyte/hour
7000
6000
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Sugar concentration mM
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2.2. Transfection of HEK293T cells and measurement of 2-deoxyglucose and GlcN transport

HEK293T cells were transfected with pcDNA3 (Invitrogen) vector containing the full-length GLUT2 cDNA as described [24]. Transport was measured as $HgCl_2$-inhibited 2-deoxyglucose (2-DOG) or GlcN uptake. Briefly, 10^5 GLUT2-expressing cells were incubated with 5 μCi of either [1,2-^{3}H]deoxy-D-glucose or D-[1-^{3}H]GlcN (NEN Life Science) diluted in cold 2-DOG or cold GlcN at final concentrations of 2 mM and 0.5 mM, respectively, for 3 min in the presence or absence of 1 mM $HgCl_2$. Cells were then washed with cold PBS containing 1 mM $HgCl_2$, lysed in 5% SDS, and counted. For normalization, the protein concentration of the cell lysate was determined by BCA assay (Pierce).

2.3. Animals

RIPGLUT1×GLUT2$^{-/-}$ mice were obtained as described previously and were from our own colony [25]. C57Bl/6J mice used as control were purchased from BRL (Switzerland).

2.4. Hepatocyte preparations

Livers from 8-week-old mice were perfused through the inferior vena cava with a buffer consisting of 140 mM NaCl, 2.6 mM KCl, 0.28 mM Na_2HPO_4, 5 mM glucose and 10 mM HEPES (pH7.4). The perfusion was first for 5 min with the buffer supplemented with 0.1 mM EGTA and then for 15 min with the buffer containing 5 mM $CaCl_2$ and 0.2 mg/ml collagenase type 2 (Worthington). All the solutions were prewarmed to 37°C and gassed with a mixture of 95% O_2/5% CO_2, resulting in a pH of 7.4. The isolated hepatocytes were filtered with nylon mesh (0.75 mm in diameter), washed twice with the perfusion buffer without collagenase, resuspended in a small volume of Dulbecco's modified Eagle's medium (DMEM; Gibco) and counted. The viability of hepatocytes was measured by trypan blue staining. The samples with viability less than 90% were discarded.

2.5. GlcN uptake study in hepatocyte

Hepatocytes were preincubated for 2 h at 37°C in DMEM in the absence of glucose but with pyruvate 1 mM and lactate 10 mM, then washed once with Krebs–Ringer buffer containing 10 mM HEPES, pH 7.4, and 1% bovine serum albumin (BSA). Uptake was initiated by diluting 20 μl of the cell suspension (25×106 cells/ml) with 230 μl of GlcN at concentrations of 500 μM and containing 1.25–5 μCi D-[1-^{3}H]GlcN per assay (1 Ci = 37 GBq). Uptake was stopped after 2 min with 1.5 ml of ice-cold PBS (10 mM $Na_2HPO_4{\cdot}2H_2O$/138 mM NaCl/2.7 mM KCl/1.76 mM KH_2PO_4, pH 7.4) containing 1 mM $HgCl_2$ (stop solution). After two washings in stop solution, the cells were lysed in 0.1% SDS, an aliquot was kept for protein determination (as above), and the rest was used for determination of radioactivity by β-counter.

3. Results

3.1. GlcN transport by GLUT1, 2 and 4 expressed in Xenopus oocytes

GLUT1, GLUT2 and GLUT4 synthetic mRNAs were injected in *Xenopus* oocytes. Glucose and GlcN uptake assays were performed 3 days later. The apparent affinity of the different GLUTs for GlcN was measured in uptake experiments performed in the presence of different concentrations of the substrate. Preliminary studies showed that uptake was linear for 45 min in the presence of 20 mM substrate. We therefore determined the uptake rates using 15 min incubation periods. Similar experiments were performed for measuring glucose uptake affinities. Fig. 1 shows the Michaelis–Menten kinetics for GlcN and glucose uptake by GLUT1, GLUT2 and GLUT4 as well as the Eadie–Hofstee transformations of these data. GlcN uptake by GLUT1 was with a K_m of 2.1 ± 0.5 mM, close to the K_m of ~3 mM for glucose. V_{max} was of 4270 ± 410 pmol/oocyte/h for GlcN, approximately 4.3-fold lower than for glucose (18 400 pmol/oocyte/h). For GLUT2, the K_m for GlcN was surprisingly low, 0.8 ± 0.1 mM, or 21-fold lower than the K_m for glucose (~17 mM), indicating a much higher apparent affinity for GlcN than for glucose. V_{max} for GlcN was 3610 ± 520 pmol/oocytes/h, ~three-fold lower than for glucose (12 000 pmol/oocyte/h). For GLUT4, the K_m for GlcN was 3.9 ± 0.3 mM, as compared to a K_m of ~6.6 mM for glucose. The V_{max} value for GlcN was 2130 ± 330 pmol/oocyte/h, 3.2-fold lower than for glucose (~6800 pmol/oocyte/h).

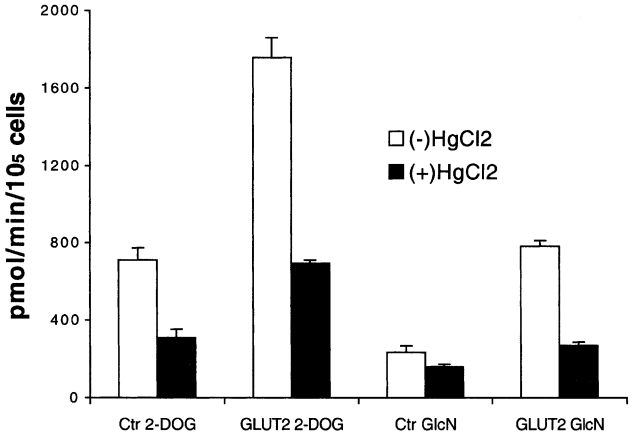

Fig. 2. GlcN and 2-DOG transport in GLUT2-transfected HEK293T cells. Uptake measurements were done in cells transiently transfected with GLUT2 or with a control (Ctr) plasmid, in the absence (blank bars) or presence (filled) of 1 mM $HgCl_2$. All data are the mean ± S.D. of three different measurements. GLUT2 expression leads to significantly increased glucose and GlcN uptake.

3.2. GlcN transport in GLUT2-transfected 293T cells

In order to check the ability of GLUT2 to transport GlcN in mammalian cells, we performed GlcN and 2-DOG uptake experiments in cells transiently transfected with GLUT2 or a control plasmid. Fig. 2 shows that specific 2-DOG uptake was increased 2.6-fold in GLUT2-expressing cells (1064 ± 94 pmol/

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Fig. 1. GlcN transport by GLUT1, 2 and 4 expressed in *Xenopus* oocytes. GLUT1, 2 and 4 were expressed from synthetic RNAs in *Xenopus* oocytes and their transport properties for GlcN and glucose evaluated. Top panel: Concentration dependence of GlcN (open circle, plain line) and glucose (filled triangles, dashed line) uptake by oocytes injected with GLUT1. Left: Data were fitted to the Michaelis–Menten equation. Each value is the mean ± S.D. of at least eight oocytes. Right: Eadie–Hofstee transformation of the uptake data. As determined in three separate experiments, K_m for GlcN was 2.1 ± 0.5 mM (V_{max}: 4270 ± 410 pmol/oocyte/h). For glucose, the data were obtained from one experiment (K_m: ~3 mM, V_{max}: 18 400 pmol/oocyte/h) and were in agreement with K_m described in the literature. Middle panel: GlcN and glucose uptake experiments performed as described above but for GLUT2-injected oocytes. Left: Michaelis–Menten fit of the uptake data. Each value is the mean ± S.D. of at least eight oocytes. Right: Eadie–Hofstee transformation of the uptake data. K_m value was 0.8 ± 0.1 mM for GlcN (V_{max}: 3610 ± 520 pmol/oocyte/h) and 17 mM for glucose (V_{max}: 12 000 pmol/oocyte/h). Lower panel: GlcN and glucose uptake experiments performed as described above but for GLUT4-injected oocytes. Left: Michaelis–Menten fit of the uptake data. Each value is the mean ± S.D. of at least eight oocytes. Right: Eadie–Hofstee transformation of the uptake data. K_m value was 3.9 ± 0.3 mM for GlcN (V_{max}: 2130 pmol/oocyte/h) and 6.6 mM for glucose (V_{max}: 6800 pmol/oocyte/h).

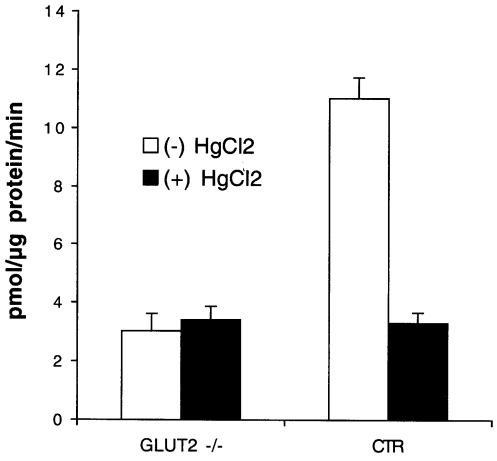

Fig. 3. GlcN uptake by control and GLUT2$^{-/-}$ hepatocytes. GlcN transport experiments were performed in the absence (blank bars) or presence (filled bars) of 1 mM $HgCl_2$. All data are the mean ± S.D. of four different uptake measurements. GLUT2 is required for GlcN uptake in hepatocytes.

min/10^5 cells vs. 402 ± 96 pmol/min/10^5 cells for GLUT2- and control-transfected cells, respectively). GlcN uptake was tested in similarly transfected cells and was 513 ± 13 pmol/min/10^5 cells for GLUT2-transfected cells as compared to 73 ± 28 pmol/min/10^5 cells for control cells. GLUT2 is thus also able to transport GlcN when expressed in mammalian cells.

3.3. GlcN uptake by control and GLUT2$^{-/-}$ hepatocytes

In order to evaluate whether GlcN is transported by GLUT2 in liver, we performed uptake experiments on hepatocytes isolated freshly from control or GLUT2-null mice [25]. As shown in Fig. 3, $HgCl_2$-inhibitable GlcN uptake was observed only in GLUT2-expressing but not in GLUT2-null hepatocytes.

4. Discussion

GlcN infusion has been widely used to activate the hexosamine pathway and GlcN administration has been shown to be beneficial for osteoarthritis. However, the mechanism by which GlcN is taken up by cells has not yet been characterized.

Here we show that GLUT1, GLUT2 and GLUT4 can transport GlcN. Furthermore, we show that GLUT2 has a very high apparent affinity for this substrate. The V_{max} for transport, however, were approximately three- to four-fold lower for GlcN than for glucose for all three transporters. We also demonstrated that GLUT2 transports GlcN when expressed in mammalian cells and that, in hepatocytes, the GlcN transport activity is exclusively accounted for by GLUT2. These data are in agreement with the fact that GLUT2 can transport NBD-GlcN (a fluorescent analogue of GlcN) when expressed in B lymphocytes [26].

GlcN concentration in the blood in basal condition is 60–80 μM [27], which is between 10- (GLUT2) and 40- (GLUT4) fold below the K_m for GlcN. However, in experimental studies GlcN is usually infused at rates giving rise to blood concentrations in the millimolar range and, when given orally to patients suffering from osteoarthritis, its blood concentration can reach 0.4 mM [21,28,29]. Exogenous GlcN administration activates the hexosamine pathway, which leads to insulin resistance and also to defects in insulin secretion. Whether these effects could also be due, to some extent, to competition between glucose and GlcN for uptake by the transporters is not known. However, if this were the case, this could be more pronounced in GLUT2-expressing cells because of this transporter's high affinity for GlcN.

Structurally, GlcN differs from glucose by an amino group replacing the hydroxyl group on carbon two (C2), which renders the molecule more hydrophobic. This decreases the V_{max} for GlcN as compared to that for glucose in a similar manner for the three GLUTs tested. The effect on substrate affinities were different, however, with GLUT2 exhibiting a markedly higher affinity for GlcN as compared to D-glucose. This differential effect on affinity, however, was lower for GLUT1 and GLUT4. GLUT2 has a K_m for 2-DOG, which lacks the hydroxyl group in position C2, of 11 mM [30], approximately 1.6-fold lower than for glucose. Therefore, it appears that, for GLUT2, a local hydrophobic environment at the C2 position of the sugar leads to increased affinity. This could be explained by better hydrophobic interaction between specific amino acids of the pore and C2 or to the ability of the amino group to form hydrogen bonds. A recent model [31] proposed that the hydroxyl group of carbon C2 of D-glucose interacts by hydrogen bond with asparagine 317 of GLUT1, which corresponds to asparagine 347 of GLUT2. The residue next to this asparagine is threonine 318 for GLUT1 and methionine 348 for GLUT2, a residue which is more hydrophobic and capable of forming a hydrogen bond. However, this residue may be located too far from the pore to interact with transported substrates [31].

The QLS motif (residues 279–281 for mouse GLUT1) has been shown to be critical for high affinity substrate recognition [32]. This motif is conserved for GLUT1, 3 and 4 but not for GLUT2, for which it is replaced by the residues HVA. These residues could also be involved in the highly different affinities of GLUT2 for glucose and GlcN.

Additional studies using different mutants of the GLUT isoforms will be required to better understand which amino acids are important for recognition of GlcN. It therefore represents a new tool for investigating the motifs implicated in substrate recognition and affinity and, more precisely, which amino acids interact with carbon C2 of glucose and its derivatives.

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